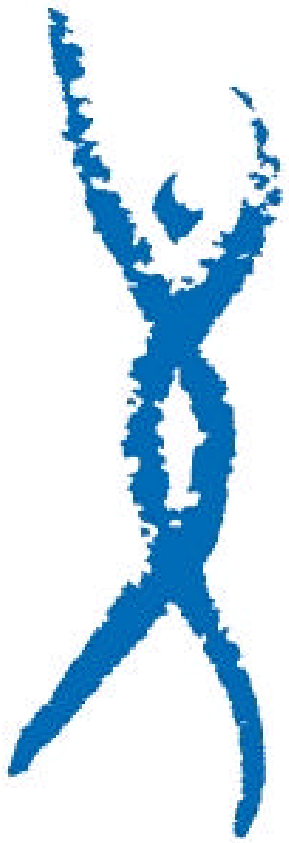


CELERA DIAGNOSTICS

A Joint Venture with Applied Biosystems

NIST Universal RNA Standards Workshop

March 29, 2003

Sheng-Yung Chang

Practical Application of Quantitative RT-PCR for *In Vitro* Human Diagnostics:

Normalization of mRNA Levels

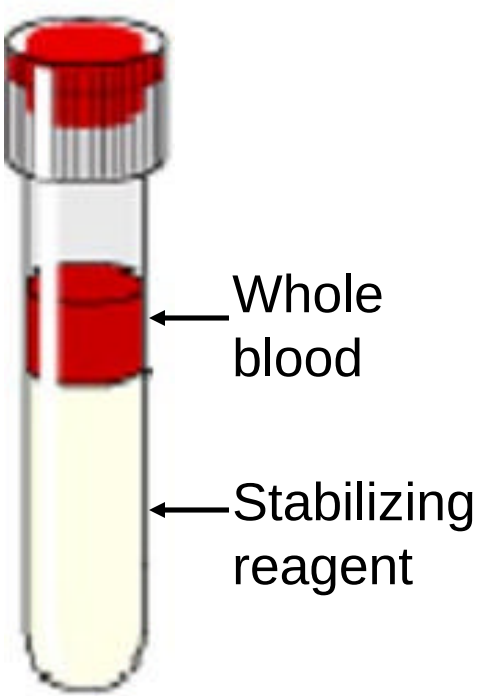
Objectives of Biomarkers in Medicine

- Identify and validate biomarker mRNA levels for:
  - Diagnosis and stage of diseases
  - Monitor disease progression
  - Monitor treatment for efficacy and toxicity

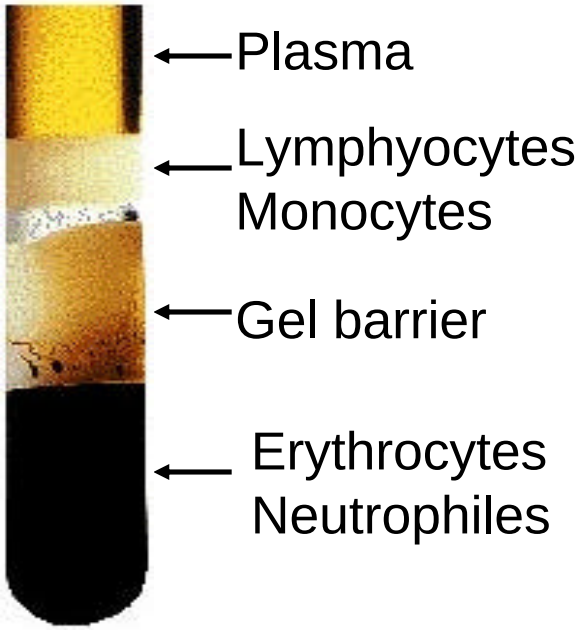
Outline

- Sample preparation
  - Blood sample collection methods
  - RNA preparation
- Quantitative RT-PCR
  - Assay format
  - Quantitation and normalization
  - Data processing
- Normal changes of mRNA profiles in healthy individuals

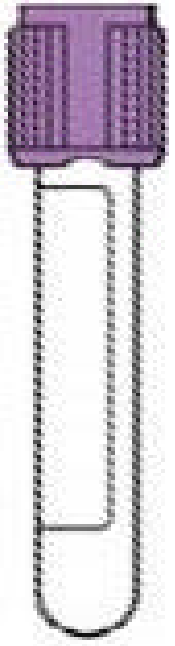
Blood Collection Tubes



PAXgene™ Blood RNA Tube



Vacutainer® CPT™



Vacutainer® Purple Top Tube

Blood Collection Tubes

	PAXgene™	Vacutainer® CPT™	Purple Top
Volume (ml)	2.5	8	3 -10
Anticoagulant	N / A	Na Citrate	K_2 EDTA
Cell Isolation	N / A	Centrifugation	Ficoll Hypaque
Cell subsets	All types	PBMCs (low % erythrocytes)	PBMCs
Cell enrichment	No	Yes	Yes
Cost	$6	$8	$0.2

Preparation of RNA Samples

Quantify RNA Concentration	OD_{260}, RiboGreen®, Agilent Bioanalyzer
Check DNA Contamination	PCR
Check RNA Integrity	Gel with SYBR® Gold, Agilent Bioanalyzer
Serial dilutions to “working” concentration	Thousand-fold dilution, from > µg / µl to < ng / µl
mRNA Profiling Experiments	Store “working” stock in poly rA

Kinetic RT-PCR

- Dye-based quantitative RT-PCR using SYBR® Green, use melting profile to confirm specificity
- Primer sets can be designed to amplify multiple variants or a specific variant
- One step RT-PCR using thermostable rTth DNA polymerase with reverse transcription step at 60°C
- ~ 0.2 ng (15-20 cells) to 2.5 ng total RNA in a 15-µl reaction in 2-4 replicates
- SYBR® Green can be replaced with Taqman probe

R. Higuchi *et. al. Bio/Technology* **11**, 1026 - 1030 (1993)
Rogge *et al. Nature Genetics* **25**, 96 101 (2000).

mRNA Profiling Using Kinetic RT-PCR

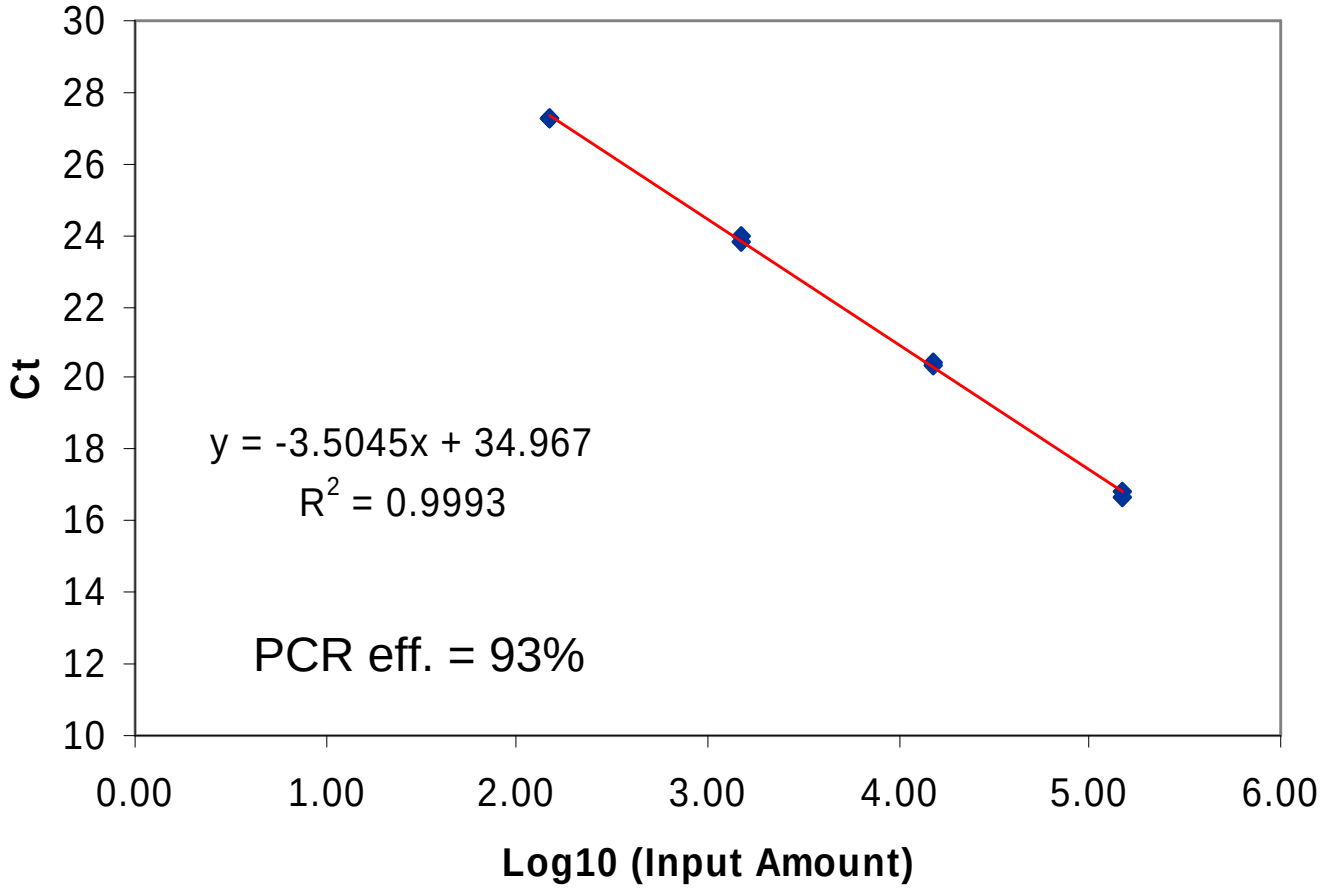
- Each experiment should include multiple levels of Quantitation Standards (QS), *in vitro* run-off RNA transcripts
  - Produce QS in large quantity with known copy number
  - Monitor reagent performance
  - Circumvent instrument-to-instrument variation
  - Convert Ct to an arbitrary unit (SGU) based on the standard curve for calculation of fold difference
- Normalizing input RNA amount
  - Select the “housekeeping” gene(s) (HSK) to normalize input RNA amount by profiling a panel of “housekeeping” genes

Profiling of "Housekeeping" Genes

- Reasons for profiling of a panel of "housekeeping" genes:
  - Quantitation of RNA can be affected by the quality of RNA and the limitation of quantitation assay
  - Serial dilution causes a small variation of input RNA amount
- "Housekeeping" gene – expressed at a relatively constant level
- HSK mRNA levels vary in different tissues, individuals, and likely different disease states
- HSK mRNA levels may also be perturbed by sample processing
- Commonly used "housekeeping" genes are not ideal
  - 18s or 28s rRNA, too abundant
  - GAPD, hypoxia inducible, up-regulated in tumor samples
  - B2M, immune responsive and interferon inducible

Warrington *et al* Physiol Genomics **2**, 143 – 147 (2000)

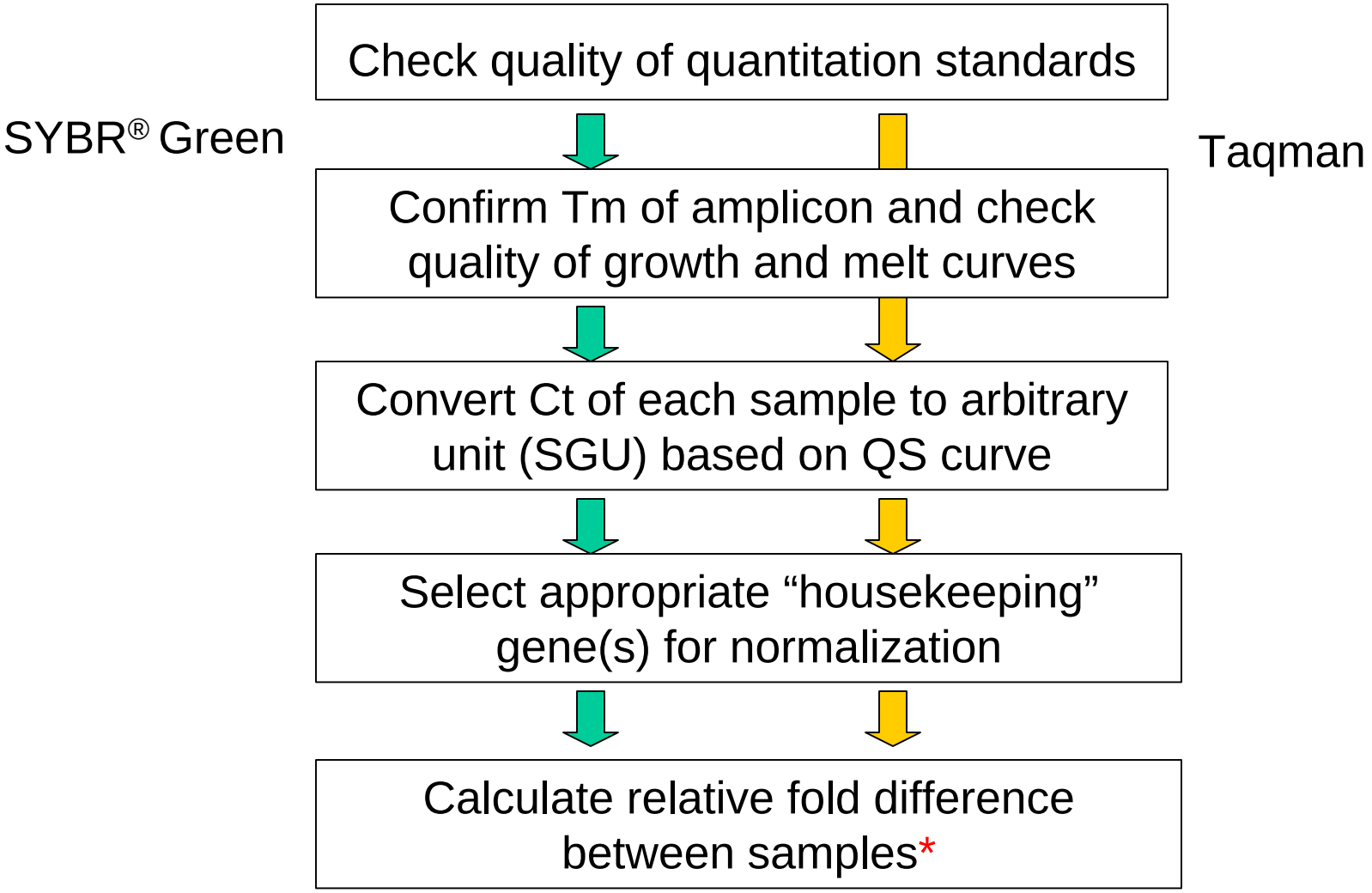
Quantitation Curve



PCR efficiency = 10^(1/-slope) – 1

- SGU in each sample is calculated based on Ct, slope, and intercept

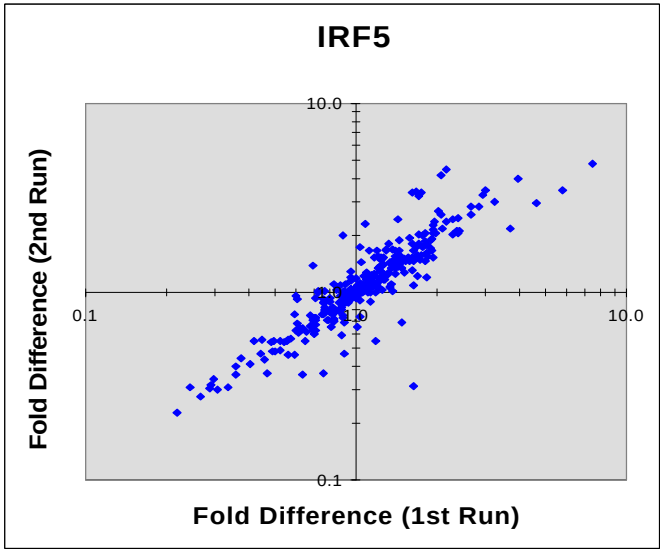
Data Processing

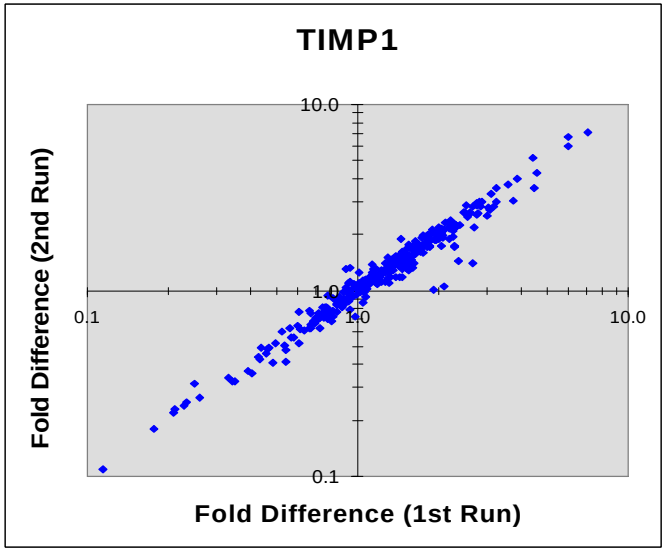


* Absolute abundance of mRNA can not be determined

Reproducibility of Fold Difference

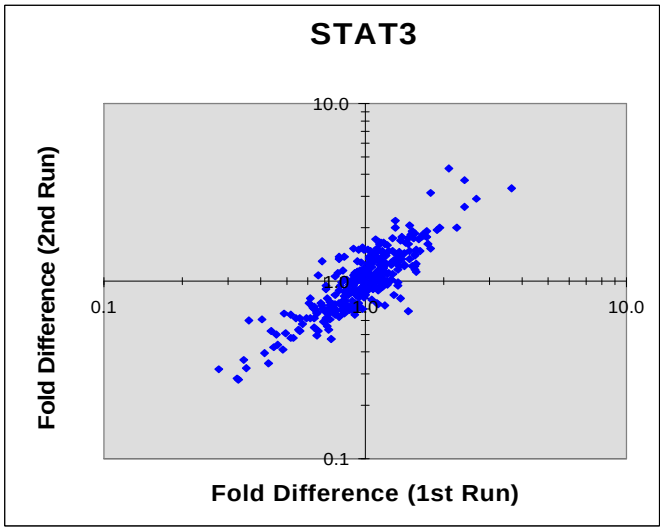
Ct:21-32

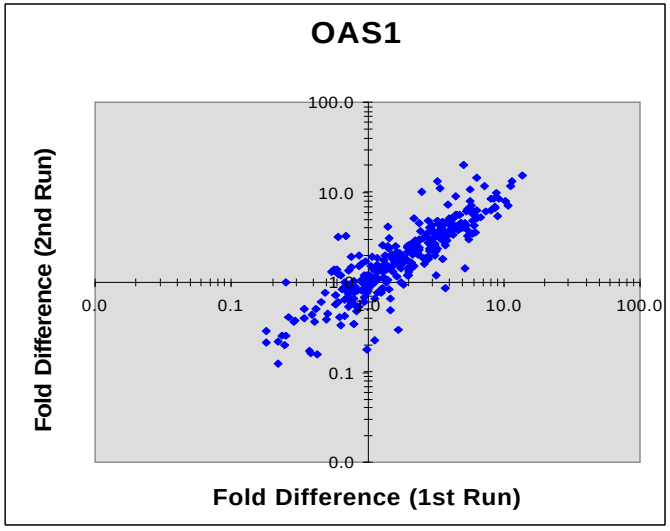




Ct:19-26

Ct:24-32





Ct:24-35

N=308 Results from two independent RT-PCR experiments

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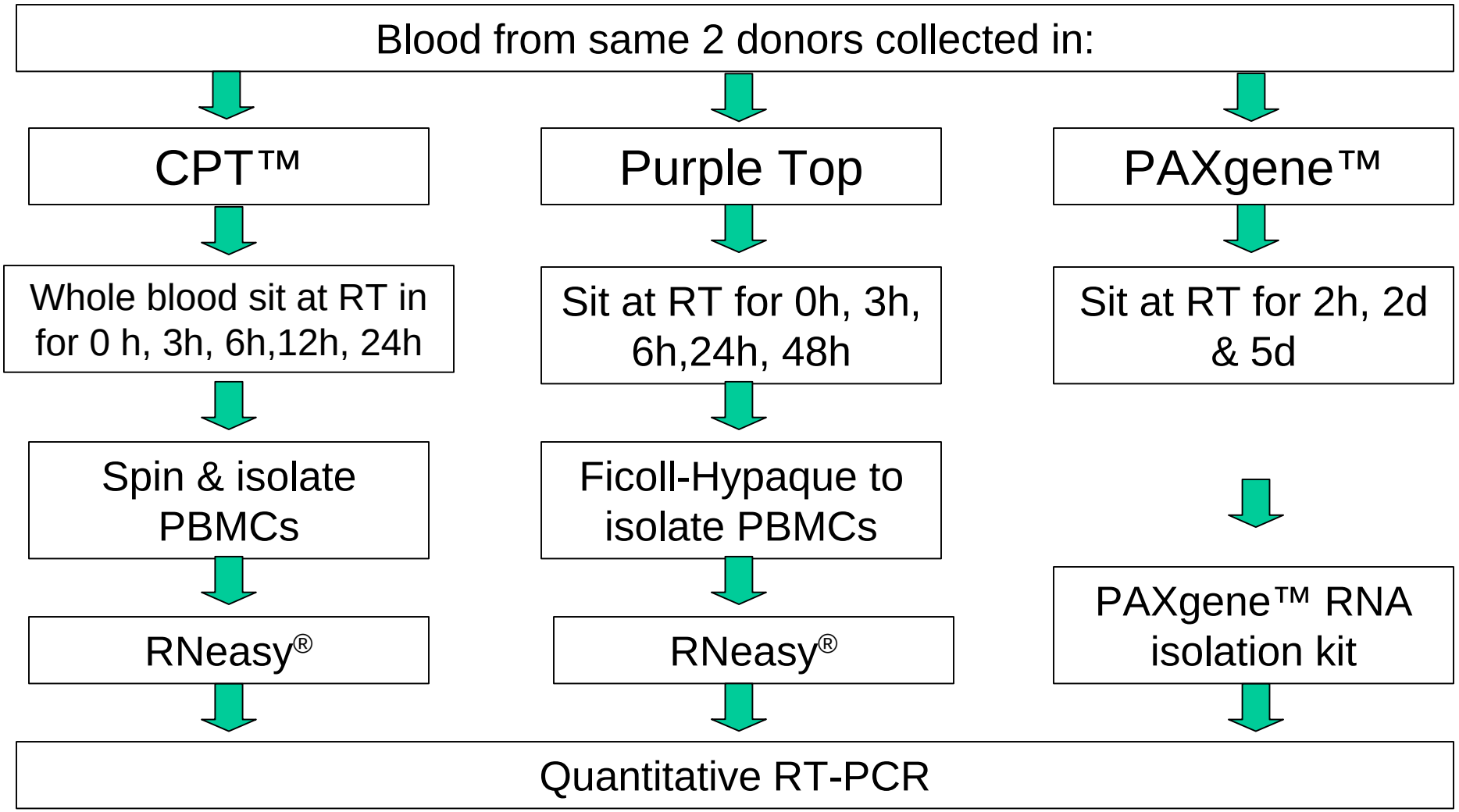
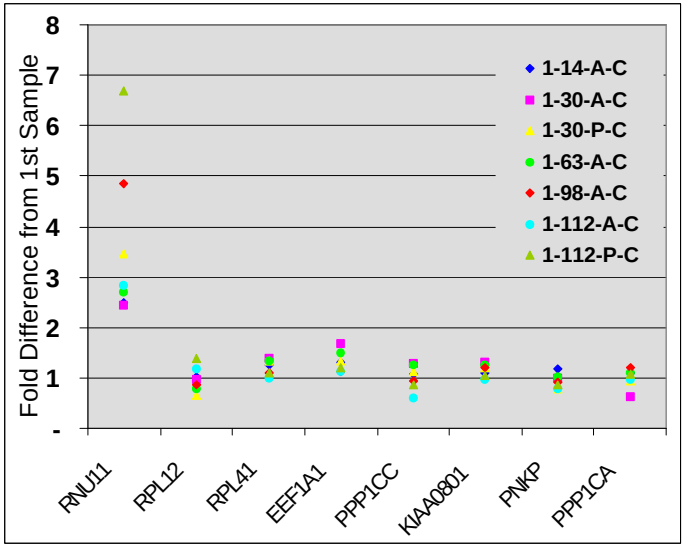
Comparison of Sample Collection Methods

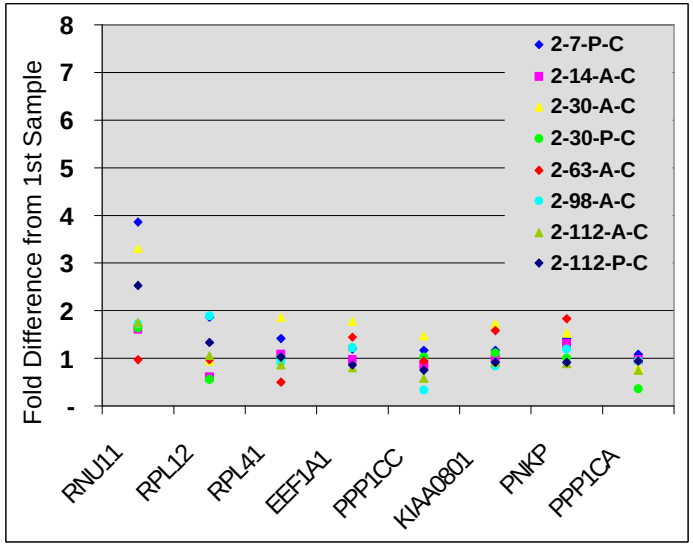


Profiling of “Housekeeping” Genes

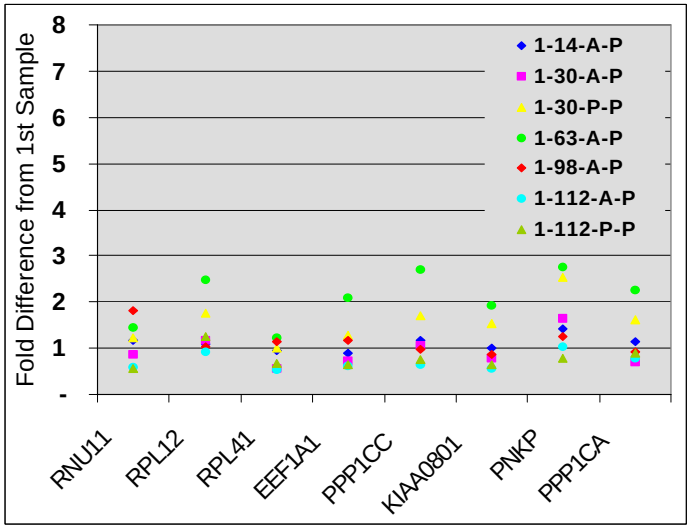
CPT™ Donor 1



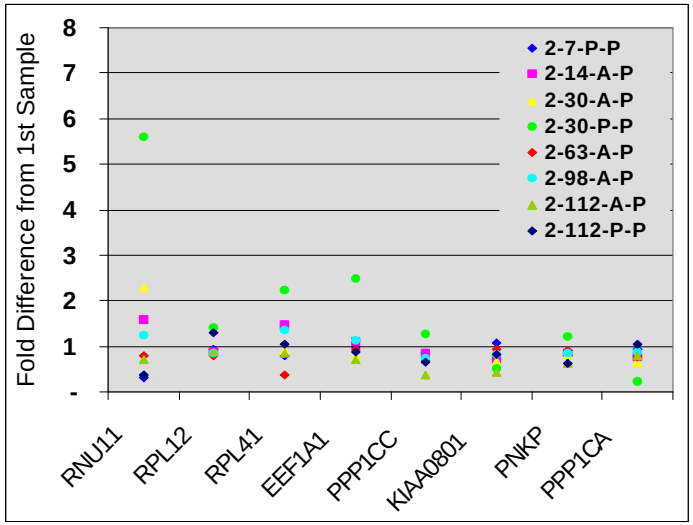
CPT™ Donor 2



PAXgene™ Donor 1



PAXgene™ Donor 2



- Divide samples into groups for “housekeeping” gene(s) selection

Determination of Normalization Factor

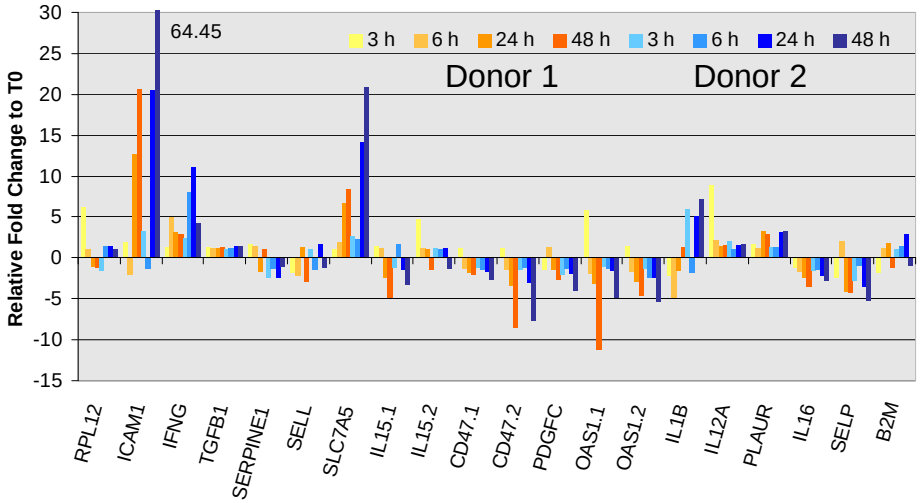
	Fold Difference (Relative to Day 0)					
	CPT™ (n=44)			PAXgene™ (n=44)		
	Ave	SD	CV	Ave	SD	CV
RNU11	2.05	1.30	63%	1.10	0.84	77%
RPL12	0.91	0.42	46%	0.95	0.37	39%
RPL41	1.05	0.30	29%	1.31	0.51	39%
EEF1A1	1.16	0.25	22%	1.19	0.46	38%
PPP1CC	1.04	0.29	28%	1.08	0.43	40%
KIAA0801	1.05	0.25	24%	0.83	0.32	39%
PNKP	1.09	0.23	21%	1.11	0.43	39%
PPP1CA	0.82	0.28	34%	0.83	0.36	43%

$$\text{HNU}^{1} = \frac{\text{SGU \# of mRNA of Interest}}{\text{Average Fold Difference of Selected HSK mRNAs}^{2}}$$

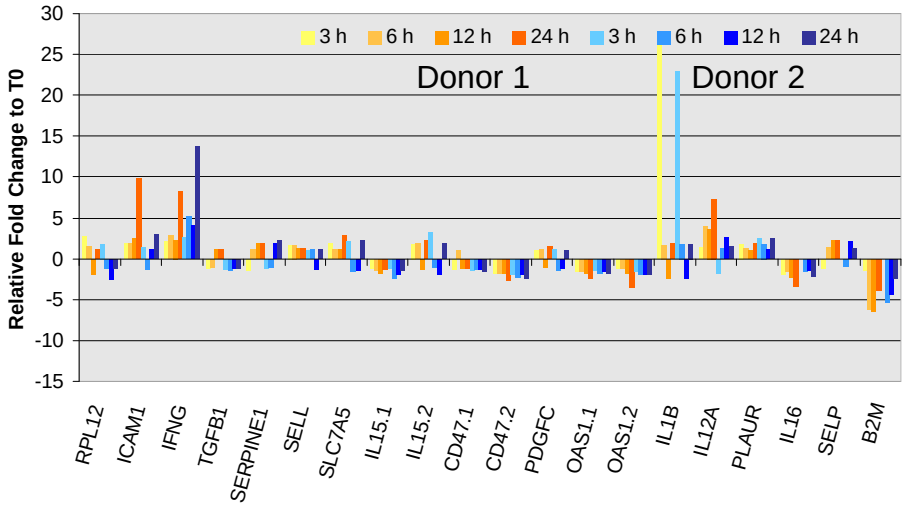
[1] **"Housekeeping" Normalized Unit** [2] **RNU11.1 was excluded**

Comparison of Blood Collection Tubes

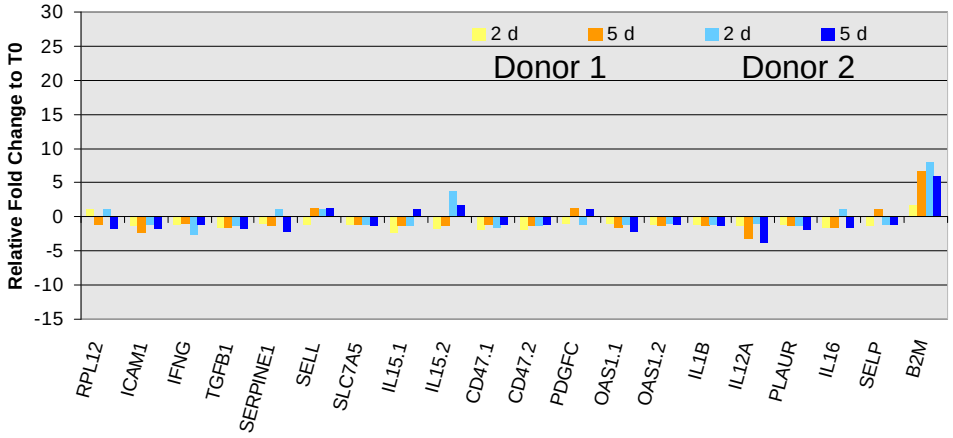
EDTA and Ficoll Hypaque



CPT™



PAXgene™



- Most mRNAs remain relatively unchanged within 6 hours in CPT™ and EDTA tubes
- PAXgene™ stabilizing reagent does not perturb mRNA profiles
- mRNA profiles in PAXgene™ samples are different because of the presence of reticulocytes and neutrophiles

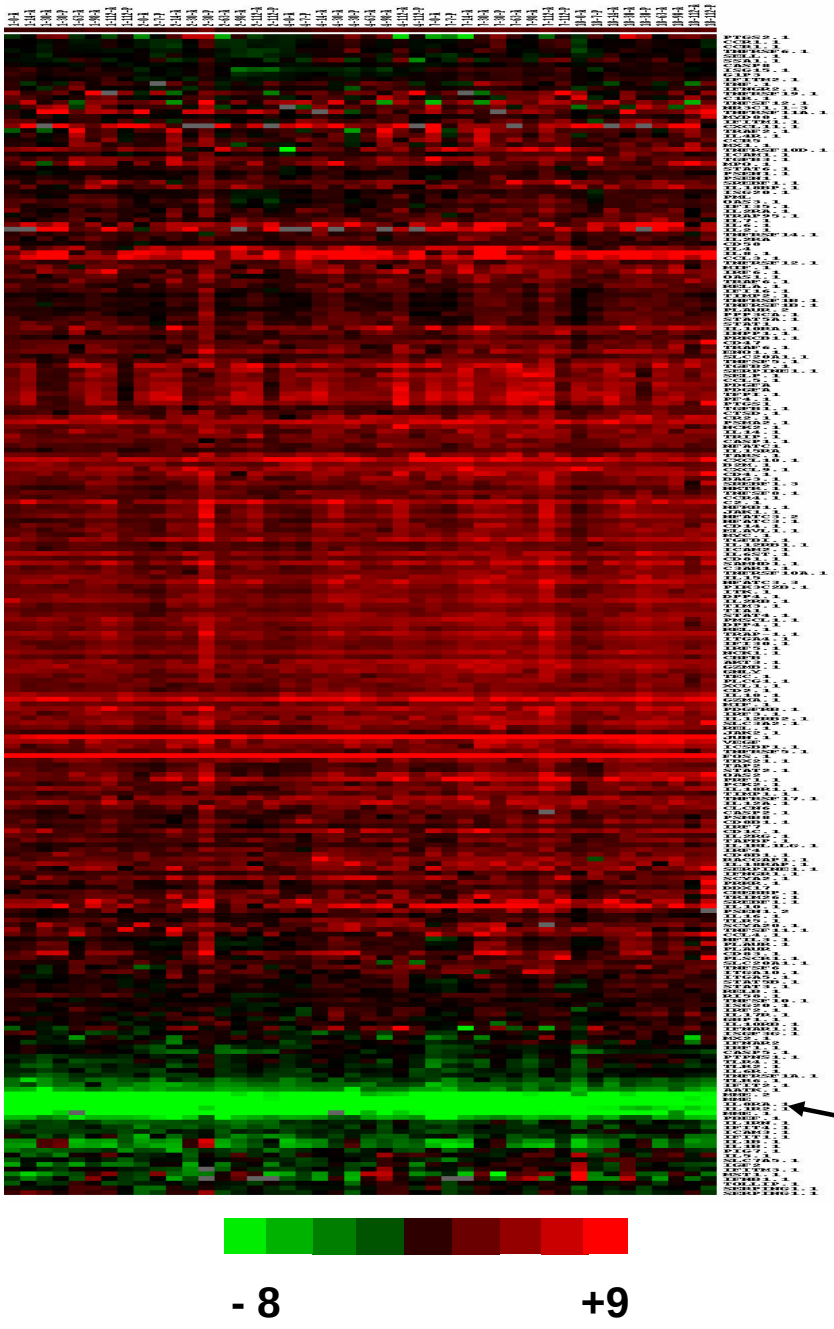
Normal Variation Study

- To examine the normal variation of mRNA profiles in blood samples
- Gene expression can be affected by circadian rhythm, diet, or environment, etc.
- Collect blood samples from 5 donors using CPT™ and PAXgene™ tubes at day 0, 7, 14, 30 AM, 30 PM, 63, 98, 112 AM, and 112 PM
- Record diet, and white blood cell counts were determined
- Profile 250 mRNAs, mostly immune response related genes

Results were presented at AACC San Diego Conference: New Technologies for Molecular Diagnostics 2002

Total RNA Recovery

	RNA Recovery (mg / ml of blood)		Hemoglobin mRNA
	CPT™	PAXgene™	Fold Difference PAXgene™ / CPT™
MAX Sample #	3.33 Donor 7 Day 63	4.92 Donor 1 Day 112 PM	962
MIN Sample #	0.95 Donor 7 Day 30 AM	0.50 Donor 2 Day 30 PM	108
AVERAGE (N=44)	1.87	2.79	274

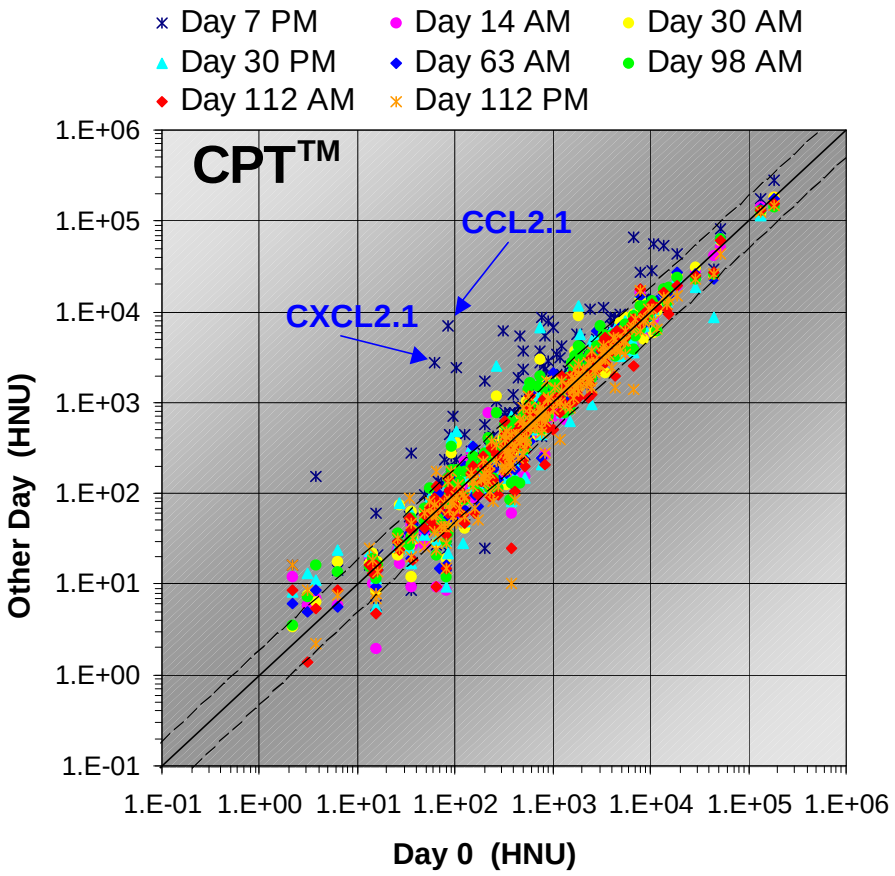


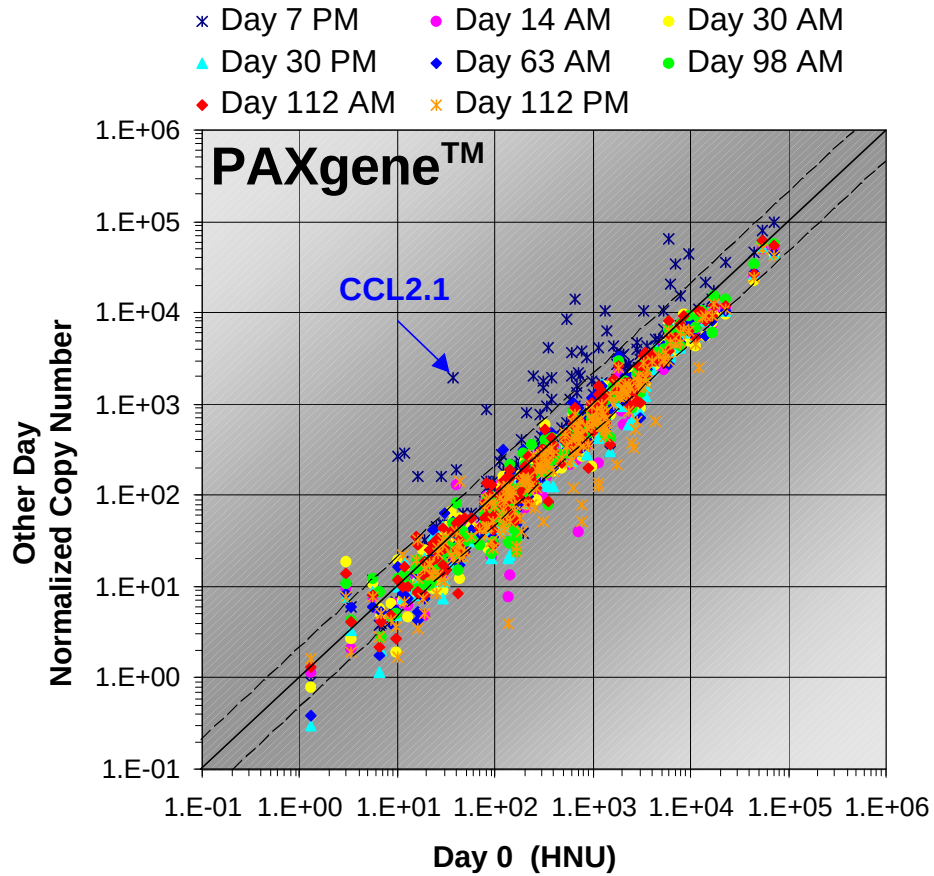
Difference of Expression Level in CPT™ and PAXgene™

Clustering of ratio of HNU in CPT sample / HUN in PAXgene™ sample

- The levels of most mRNAs were higher in CPT samples
- The level of IL1R2 in CPT samples was 6- to 50-fold lower
- The ratio was likely affected by the presence of reticulocytes and neutrophils in PAXgene™ samples

Intra-Donor Variation

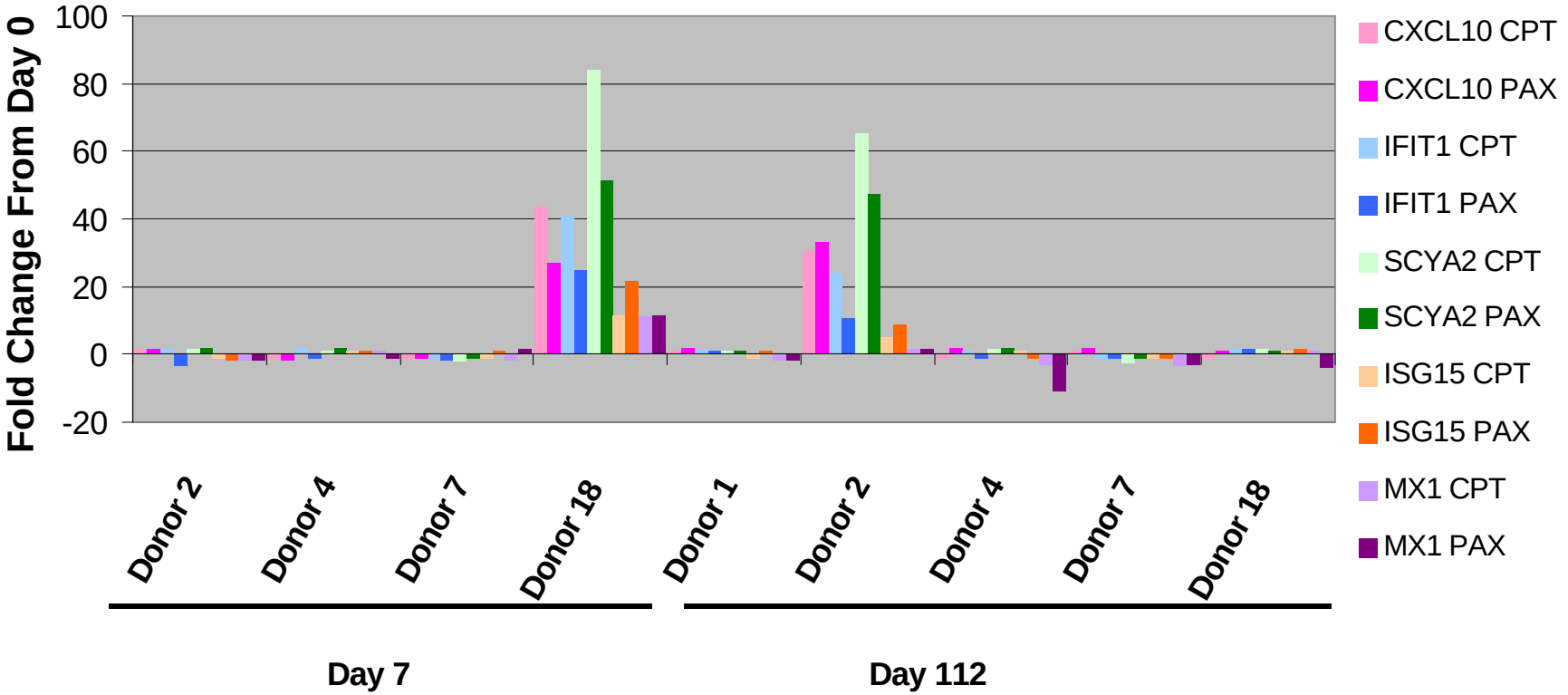




Donor 18

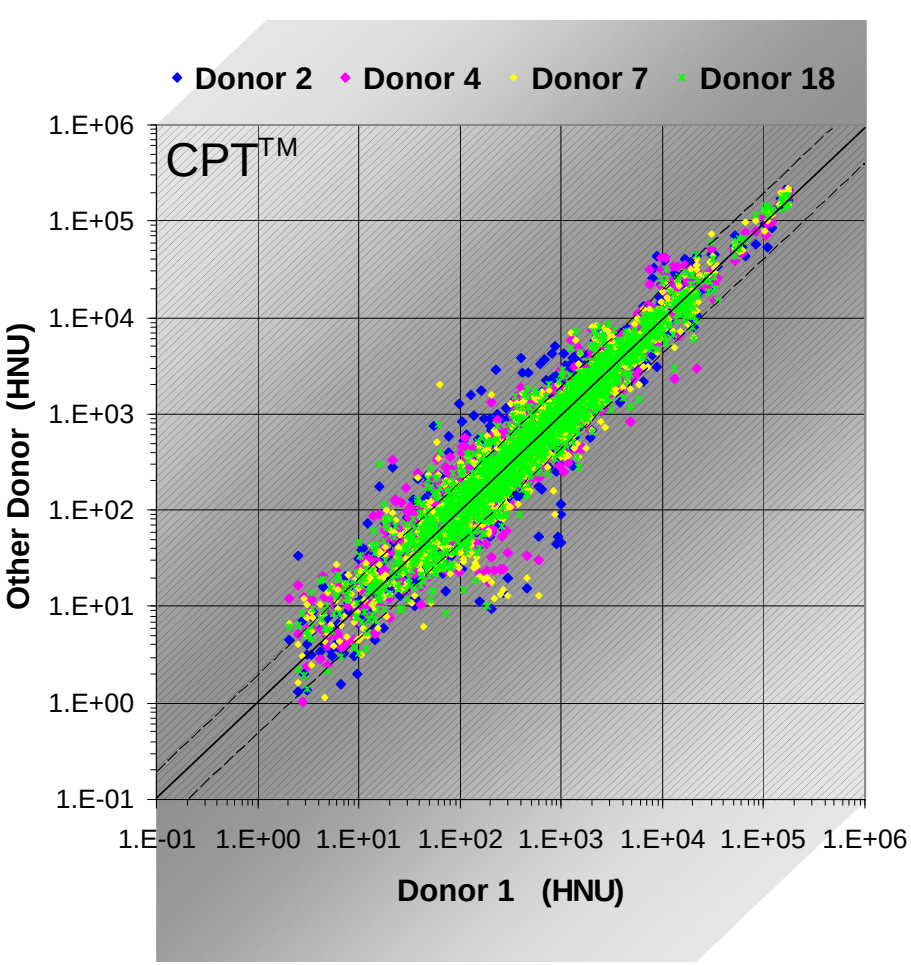
- Most of mRNAs varies less than 2-fold

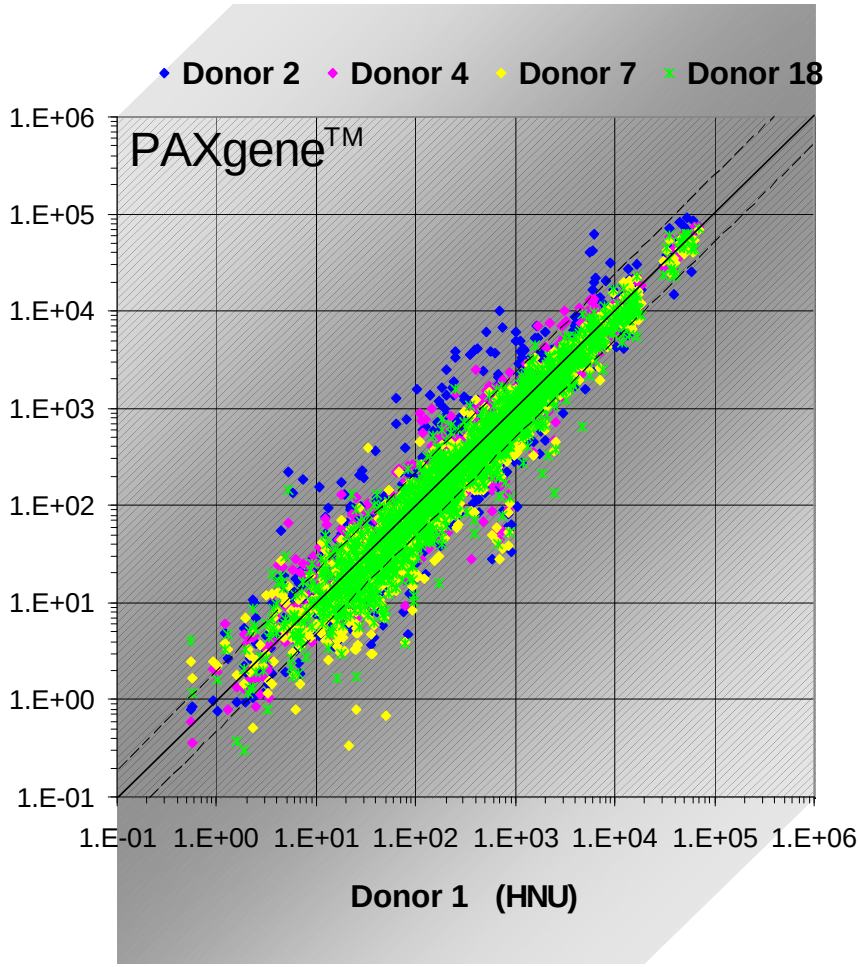
Unique Gene Expression In Donors 2 and 18



- Both donors 2 and 18 are females
- SCYA2 (CCL2), a.k.a monocyte chemoattractant protein-1 – recruit macrophages to corpus luteum during menstrual cycle
- IFIT1, CXCL10, ISG15, and MX1 are IFN inducible genes

Inter-Donor Variation





- The variation of mRNA profiles are greater between individuals
- Fold differences vary from – 140-fold to + 800-fold

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Summary

- mRNA profiles are easily perturbed by sample collection and processing methods
- The impact of the presence of abundant hemoglobin mRNA in sample collected with PAXgene™ tubes on gene expression studies is not clear yet
- Standardized sample collection and processing method should be implemented in all clinical studies
- Approximately 13 % of mRNAs vary more than 2-fold in normal conditions
- Identification of biomarkers using blood samples is likely feasible